Microarray-based analysis of cadmium-responsive microRNAs in rice (*Oryza sativa*)

Yanfei Ding¹², Zhen Chen¹ and Cheng Zhu²*

¹ State Key Laboratory of Plant Physiology and Biochemistry, College of Life Sciences, Zhejiang University, Hangzhou 310029, China
² Zhejiang Provincial Key Laboratory of Biometrology and Inspection and Quarantine, College of Life Sciences, China JiLiang University, Hangzhou 310018, China

* To whom correspondence should be addressed. E-mail: pzhch@cjlu.edu.cn

Received 25 August 2010; Revised 14 January 2011; Accepted 3 February 2011

Abstract

MicroRNAs (miRNAs) are a class of small non-coding RNAs that negatively regulate specific target mRNAs at the post-transcriptional level. Plant miRNAs have been implicated in developmental processes and adaptations to environmental stresses. Cadmium (Cd) is a non-essential heavy metal that is highly toxic to plants. To investigate the responsive functions of miRNAs under Cd stress, miRNA expression in Cd-stressed rice (*Oryza sativa*) was profiled using a microarray assay. A total of 19 Cd-responsive miRNAs were identified, of which six were further validated experimentally. Target genes were also predicted for these Cd-responsive miRNAs, which encoded transcription factors, and proteins associated with metabolic processes or stress responses. In addition, the mRNA levels of several targets were negatively correlated with the corresponding miRNAs under Cd stress. Promoter analysis showed that metal stress-responsive *cis*-elements tended to occur more frequently in the promoter regions of Cd-responsive miRNAs. These findings suggested that miRNAs played an important role in Cd tolerance in rice, and highlighted a novel molecular mechanism of heavy metal tolerance in plants.

Key words: Cadmium, gene regulation, heavy metal tolerance, microarray, microRNA, rice, stress response.

Introduction

Cadmium (Cd) is a widespread heavy metal pollutant in the environment, originating mainly from industrial processes and phosphate fertilizers (Pinto et al., 2004). Cd can be easily taken up by plants, resulting in various toxicity symptoms, such as chlorosis, wilting, growth reduction, and cell death (Sandalio et al., 2001; Rodriguez-Serrano et al., 2009). The cellular toxicity caused by Cd might result from interactions with the carboxyl or thiol groups of proteins (Sanitá di Toppi and Gabbrielli, 1999) and the generation of reactive oxygen species (ROS), inducing oxidative stress (Schützendübel et al., 2001; Schützendübel and Polle, 2002). Plants possess a range of mechanisms involved in Cd detoxification, including metal transport, chelation, and sequestration (Clemens, 2001; Hall, 2002). For example, the natural resistance-associated macrophage protein (Nramp) transporter was reported to function in Cd uptake in *Arabidopsis thaliana* (Thomine et al., 2000), whereas the intracellular chelation of Cd by glutathione (GSH) and phytochelatins (PCs) represented a ubiquitous detoxification strategy adopted by many plant species (Cobbett and Goldsbrough, 2002). Detoxification can also be achieved by proline synthesis (Siripornadulsil et al., 2002). Although some of the molecules involved in Cd tolerance have been identified, the regulatory mechanisms involved are still largely unknown.

Recently, microRNAs (miRNAs), a large family of endogenous small RNAs, were reported to have crucial
roles in the modulation of gene expression (Carrington and Ambros, 2003; Bartel, 2004). miRNAs arise from hairpin-structured precursors after two sequential cleavages carried out by DICER-LIKE1 (DCL1) in plants, or Drosha and Dicer in animals (Reinhart et al., 2002; Bartel, 2004; Jones-Rhoades et al., 2006). Mature miRNAs are incorporated into the RNA-induced silencing complex (RISC) to negatively regulate the expression of specific mRNA targets through mRNA cleavage, decaying, or translational repression. In plants, miRNAs generally interact with their targets through perfect or near-perfect complementarity and lead to target mRNA cleavage (Rhoades et al., 2002; Carrington and Ambros, 2003; Schwab et al., 2005). Functional studies showed that miRNAs are implicated in most of the essential physiological processes in plants, including organ development, signal transduction, and the stress response (Aukerman and Sakai, 2003; Palatnik et al., 2003; Guo et al., 2005; Sunkar and Zhu, 2004; Lu et al., 2005).

Abiotic stresses, such as drought, cold, and heavy metals, cause a rapid and excessive release of ROS in plant cells (Apel and Hirt, 2004). Evidence also suggested that miRNAs in plants responded to abiotic stresses (Sunkar and Zhu, 2004; Lu et al., 2005). For example, miR395 and miR399 were induced by sulphate and phosphate starvation, respectively (Jones-Rhoades and Bartel, 2004; Fuji et al., 2005; Chiou et al., 2006): miR395 participated in sulphate assimilation and allocation (Jones-Rhoades and Bartel, 2004), and miR399 was crucial in maintaining phosphate homeostasis (Fuji et al., 2005; Chiou et al., 2006). Research also indicated that miR168, miR171, and miR396 were responsive to high salinity, drought, and cold stress in Arabidopsis (Liu et al., 2008). Several abiotic stresses, including excess heavy metals, led to oxidative stress by generating ROS in plants (Dietz et al., 1999), and miRNAs were shown to be important for plant responses to heavy metal stress (Sunkar and Zhu, 2004; Sunkar et al., 2006; Ding and Zhu, 2009). Northern blot analysis showed that the expression of miR398 in Arabidopsis was downregulated by heavy metals, such as copper (Cu) and iron (Fe), and was important for accumulation of Cu, Zn-superoxide dismutase (CSD), a scavenger of superoxide radicals (Sunkar et al., 2006). Through PCR-based analyses, miR393 and miR171 responded to heavy metals in Brassica napus (Xie et al., 2007; Huang et al., 2010) and Medicago truncatula (Zhou et al., 2008). Additionally, Huang et al. (2009) isolated 19 potential novel miRNAs that were responsive to Cd by using conventional sequencing approaches. However, the methods used so far for identifying metal-responsive miRNAs [i.e. northern blot, reverse transcription-PCR (RT-PCR), and conventional sequencing] had several disadvantages, such as not being sensitive enough to identify miRNAs at low expression levels, and not suitable for large-scale detection, while microarray analysis is one of the most widely used strategies for the high-throughput analysis of miRNA expression patterns. By using microarray technology, it is possible to carry out a large-scale survey of the expression patterns of all the annotated miRNAs in a given plant species (Zhao et al., 2007; Liu et al., 2008).

Rice (Oryza sativa) is a major cereal crop and produces food for many populations of the world. However, Cd pollution in the soil affects the rice yield and grain quality in many areas. Several rice miRNAs have been reported to play roles in responses to oxidative stresses, such as drought stress (Zhao et al., 2007). However, knowledge of the role of miRNAs in response to Cd stress in rice is still limited. In this study, 19 Cd-responsive miRNAs were identified in rice by using miRNA microarray analysis, of which six were further validated experimentally by quantitative real-time PCR (qPCR); results from both experiments were consistent. The fine-scale expression analysis of Cd-responsive miRNA targets and the search for metal-responsive elements (MREs) in the miRNA promoters provided molecular evidence for the potential involvement of certain miRNAs in Cd tolerance in rice. Together, the identification of Cd-responsive miRNAs and their targets could help to uncover the molecular mechanisms of Cd tolerance in plants.

Materials and methods

Rice growth and treatment

Wild-type rice Zhonghua 11 (O. sativa L. subsp. japonica) seeds were sterilized with 3% sodium hypochlorite for 20 min and washed thoroughly with distilled water. After germination at 30°C in the dark, the seeds were grown under a 13 h light (29°C)/11 h dark (22°C) photoperiod. For Cd stress experiments, 7-day-old seedlings were exposed to 60 μM CdCl2 for 24 h. Roots were harvested as a pool for each sample at 0, 3, 6, 12, and 24 h after Cd treatment. Seedlings not treated with Cd served as controls. Total RNA was extracted from 7-day-old rice roots with Trizol reagent (Invitrogen).

Microarray assay analysis

The miRNA microarray assays were performed by a service provider, LC Sciences (http://www.lcsiences.com). Chip hybridization experiments were carried out in duplicate initiated from different biological samples. Based on mature miRNA sequences of rice downloaded in miRBase Release 11.0 (http://microrna.sanger.ac.uk/), probes were designed to detect Cd-inducible rice miRNAs on microarray chips. The microarray contained 115 probes that were complementary to the miRNAs in rice. Each probe was repeated three times on the chip to ensure microarray reproducibility. For microarray quality control, SS rRNA was designed as the inner positive control; blank and non-homologous nucleic acids served as negative controls. Sufficient RNA for two biological replicates was extracted to run the microarray experiments.

Total RNA from rice roots treated with 60 μM CdCl2 for 6 h and of the control was isolated with Trizol reagent (Invitrogen) and size fractionated using a YM-100 Microcon centrifugal filter (Millipore). The small RNAs (<300 nucleotides) were 3′-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining. The purified small RNAs were labelled with Cy3 (control) and Cy5 (treated) fluorescent dyes. Hybridization was performed overnight on a μParaFlo microfluidic chip using a microcirculation pump (Atactic Technologies). The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization used 100 μl of 6× SSPE buffer (0.90 M NaCl, 60 mM Na2HPO4, 6 mM EDTA, pH 6.8) containing 25% formamide at 34°C. After hybridization, signals were detected using tag-specific Cy3 and Cy5 dyes. Hybridization images were collected using a laser scanner (GenePix 4000B, Molecular Device).
and digitized using Array-Pro image analysis software (Media Cybernetics).

Data were analysed by subtracting the background and then normalizing signals using a LOWESS filter (locally weighted regression). For the two-colour experiments, the ratio of the two sets of detected signals (log$_2$ transformed and balanced) and t-test P-values were calculated; differentially detected signals werethose with P-values <0.01. To identify Cd-induced miRNAs, a criterion of fold change >1.5 and P-values <0.01 was used.

Quantitative real-time PCR assay

The time course of miRNA expression was examined by qPCR experiments. Total RNA was isolated from roots of controls and treated plants at 0, 3, 6, 12, and 24 h after Cd exposure and treated with 5 U of RNase-free DNase I (TaKara) to remove DNA contamination from the RNA. Real-time PCRs were carried out on a LightCycler480 machine (Roche, Shanghai, China) (parameters: 95 °C for 1 min, followed by 45 cycles of 95 °C for 10 s, 58 °C for 15 s, and 72 °C for 15 s). All reactions were done in triplicate. U6 was chosen as the control for small RNAs. The stem-loop reverse transcription primers were designed following the method described by Chen et al. (2005). Briefly, six nucleotide tips pairing with the mature miRNA 3' end were linked to a self-looped sequence (GTCGCTTCAGGTCCGTGTCGAGTCGGAAATGCTGCACTGGATAGCAC) to make up the stem-loop reverse transcription primer. The primer was hybridized to a miRNA molecule and then reverse transcribed with PrimeScript reverse transcriptase (TaKara, Japan). PCR primers, including a miRNA-specific forward primer and a reverse primer, were then added to amplify the PCR products. The sequences of stem-loop reverse transcription primers and miRNA-specific PCR primers are listed in Supplementary Table S2 available at JXB online.

The expression analysis of several target genes was also performed by qPCR. DNase I-treated RNA was reverse transcribed using an oligo(dT) primer and a PrimeScript™ RT reagent kit (TaKara, Japan) to generate cDNA. The target gene primers were then added to perform the PCR. Real-time PCR was carried out using SYBR Premix Ex Taq™ (TaKara, Japan) for detection of PCR products. Quantification of gene expression was done using the comparative CT method. Experiments were performed in triplicate and the results were presented by means ±SE of three replicates. β-Actin was chosen as a reference gene. The primer pairs for the amplification of β-actin and several target genes were as follows: β-actin forward, 5'-GCGGTCCTTCCCTCTGTATGC-3'; reverse, 5'-GGGGACAG TGTGGCTGAC-3'; AGO forward, 5'-CCCCAAGGACCGAGA CCAG-3'; reverse 5'-GGGGCAACTAGAAGCGAGG-3'; HD-Zip forward, 5'-ATGGCTAAGATCTCCCGAG-3'; reverse, 5'-CAGACGGAAAATACCTCA-3'; and RLK forward, 5'-CCTTTG CAAACTTTCCCG-3'; reverse, 5'-ACTGCTCTTCTCTCA ACA-3'.

Semi-quantitative RT-PCR analysis

Total RNA was extracted from 7-day-old seedlings using Trizol reagent (Invitrogen). Total RNA was reverse transcribed using an oligo(dT) primer and PrimeScript reverse transcriptase (TaKara) according to the supplier’s manual. Primers for the six precurso(miRNAs were then added to perform the PCR. β-Tubulin was used as the inner control for RT-PCR. RT-PCR conditions for β-tubulin amplification were as follows: 94 °C for 10 min, 25 cycles (94 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min), then 72 °C for 10 min. Primers used for β-tubulin were as follows: forward, 5'-ACTGGTTCTGTTATGGA-3'; and reverse, 5'-TAGTG TGGCATTGAAGT-3' (135 bp). The primer pairs for the amplification of pre-miRNAs are shown in Supplementary Table S3 at JXB online.

miRNA target prediction

Plant miRNAs complement their target mRNAs by perfect or near-perfect base pairing. Based on a sequence similarity search, a web-based computing system, miRU (http://bioinfo3.noble.org/miRNA/miRU.html) (Zhang, 2005), was used to predict target miRNAs for the Cd-responsive miRNAs by mature miRNA sequences. The miRU program reports all potential sequences, with mismatches no more than specified for each mismatch type. The minimal score among all 20-mers cannot exceed 3.0 with default parameters. The conservation of target complementarity in other plant species was also used for identification of miRNA targets and further reduction of false positives. The functions of target genes were obtained from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/index.shtml).

miRNA promoter selection and cis-acting element analysis

Promoters of osa-miRNAs were recruited mainly by following the rules discussed by Zhou et al. (2007) and Meng et al. (2009). Pre-miRNA sequences were downloaded from miRBase, miRBase Release 11.0 (http://microrna.sanger.ac.uk/). First, if a pre-miRNA and its closest upstream gene were unidirectional (i.e. the same direction) and the distance between them was >2400 bp, the 2000 bp sequence upstream of the pre-miRNA was retrieved. If this was not the case, the sequence between the site 400 bp downstream of the upstream gene and the miRNA precursor was used. Secondly, if a pre-miRNA and its closest upstream gene were convergent (i.e. the opposite direction) and the distance between them was >4000 bp, the 2000 bp sequence upstream of the precursor was retrieved. If this was not the case, the sequence from the pre-miRNA to the middle point between the upstream gene and the pre-miRNA was obtained. These obtained sequences were then checked by using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/) (Lescot et al., 2002), a database of plant cis-acting regulatory elements and a portal for tools for the in silico analysis of promoter sequences. Metal stress-related cis-elements were recruited, and the distribution patterns were also characterized.

Results

Identification of Cd-responsive miRNAs in rice

In this study, miRNA microarrays were used to investigate the expression patterns of miRNAs in rice seedlings under Cd stress (Fig. 1, and Supplementary Table S1 at JXB online). In general, many miRNAs showed significant alterations in expression in response to Cd treatment. Nineteen miRNAs belonging to 10 families were identified to be responsive to Cd. It was found that miRNA members of the same family had similar expression profiles, probably owing to highly homologous sequences, which were difficult to distinguish even by using hybridization-based methods. Among the 19 Cd-responsive miRNAs, only miR528 was significantly up-regulated; the other 18 miRNAs (miR162a, miR168a, miR168b, miR166m, miR166i, miR166e, miR166k, miR166g, miR171b, miR171a, miR171g, miR396d, miR390, miR156l, miR156k, miR156a, miR1432, and miR444b.1) were down-regulated by Cd stress. These down-regulated miRNAs belong to the miR162, miR168, miR166, miR171, miR396, miR390, miR156, miR1432, and miR444 families (Table 1).

Fine-scale validation and time-course analysis of the Cd-responsive miRNAs

To validate the microarray results, six rice Cd-responsive miRNAs were selected for further experimental
confirmation: miR162a, miR166m, miR171b, miR390, miR168b, and miR156l (Fig. 2). Their expression levels were measured by using qPCR. Semi-quantitative RT-PCR analysis was also performed to detect the expression of their pre-miRNAs (precursor miRNAs) (detailed in Supplementary Fig. S1 at JXB online). Both the qPCR and the RT-PCR results showed that all of these six miRNAs were down-regulated after exposure to Cd for 6 h, which was consistent with the microarray data.

Temporal expression patterns of the six validated miRNAs were detected at different time points of Cd treatment by qPCR (Fig. 2). Seven-day-old rice seedlings were exposed to 60 μM CdCl₂ for 24 h. RNA was isolated from the treated rice roots and control at 0, 3, 6, 12, and 24 h after Cd treatment. Real-time PCR analysis demonstrated that these miRNAs showed different expression patterns during Cd treatment. In rice roots treated with 60 μM Cd, miR162a, miR168b, and miR390 started to decrease within 3 h and were down-regulated at 24 h, while miR171b was up-regulated after 3 h, then down-regulated from 6 h to 24 h after exposure to Cd. The expression of miR166m and miR156l was down-regulated from 3 h to 12 h after Cd treatment, and then recovered to normal levels during later stages. In general, it was shown that expression of these six miRNAs from roots treated with Cd decreased significantly during the course of treatment compared with the control. These results suggested the high sensitivity of these miRNAs to Cd stress in rice.

Prediction of miRNA targets
To learn more about the roles of the miRNAs in Cd tolerance, potential miRNA targets were identified by using
miRU and BlastN searches (Rhoades et al., 2002; Zhang, 2005). One interesting observation was that many of the predicted target genes of the Cd-responsive miRNAs encode transcription factors belonging to various families (Table 2).
Five miRNA families (miR166, miR171, miR396, miR156, and miR444), whose target genes encode transcription factors, were all down-regulated in response to Cd exposure. This indicated that their corresponding transcription factor genes were activated, which, in turn, regulated the expression of specific protein-encoding genes and led to enhanced Cd tolerance. miR166 was predicted to target homeodomain-leucine zipper (HD-Zip) transcription factors, which had been characterized as being crucial for lateral root development and leaf polarity in Arabidopsis (Hawker and Bowman, 2004; Jones-Rhoades and Bartel, 2004). Moreover, the HD-Zip transcription factors in Arabidopsis were reported to be regulated by water deficit, osmotic stress, and exogenous abscisic acid (ABA) treatments (Henriksson et al., 2005). miR171 was found to target Scarecrow-like transcription factors controlling floral development (Reinhart et al., 2002; Rhoades et al., 2002). miR444b.1 was predicted to target MADS-box transcription factors, which had a broad spectrum of biological functions, including coping with salinity and cold stress conditions (Kuo et al., 1997; Lozano et al., 1998).

The results also showed that several target genes of the Cd-responsive miRNAs encode proteins that function in diverse biological processes. For example, EF-hand proteins and leucine-rich repeat (LRR) receptor protein kinases were reported to be involved in signal transduction as well as in external or endogenous stimulus responses (Tichtinsky et al., 2003; Osakabe et al., 2005; Lu et al., 2008). DCL1 and ARGONAUTE (AGO) proteins, which were indispensable factors during miRNA processing and functioning, were predicted to be targeted by miR162 and miR168, respectively. These results suggested that feedback regulation played a role in miRNA activity under Cd-stressed conditions in rice.

Expression analysis of target genes
To investigate whether the predicted target genes were actually regulated by Cd-responsive miRNAs, the expression levels of three targets [AGO (the target of miR168), HD-Zip (the target of miR166), and RLK (the target of miR390)] were measured in rice seedlings exposed to 60 μM Cd from 0 h to 24 h by using qPCR. As shown in Fig. 3A and B, the abundance of AGO and HD-Zip transcripts increased, whereas the expression of miR168 and miR166 was inhibited from 3 h to 24 h after exposure to Cd. Additionally, changes in miR390 abundance had a negative effect on the abundance of RLK at the four treatment time points during Cd stress (Fig. 3C). The profiles of miRNAs (miR168, miR166, and miR390) and target transcripts (AGO, HD-Zip, and RLK) were complementary, but not exactly opposite, to each other (i.e. the time point showing the higher level of miRNA accumulation did not coincide with the lower accumulation of target mRNA). Together, the negatively correlated expression patterns between miRNAs and their targets further validated the regulatory role of miRNAs on their targets, and indicated the role of miRNAs in Cd tolerance of rice seedlings.

**Table 2.** Predicted targets of Cd-responsive miRNAs and their function annotations

<table>
<thead>
<tr>
<th>MiR ID</th>
<th>Targets</th>
<th>Target function</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR162a</td>
<td>DCL1</td>
<td>miRNA processing</td>
</tr>
<tr>
<td>miR528</td>
<td>OsDCL1</td>
<td>miRNA processing</td>
</tr>
<tr>
<td>miR168b/a</td>
<td>ARGONAUTE protein</td>
<td>miRNA processing; plant development</td>
</tr>
<tr>
<td>miR166b/i/m/k/g</td>
<td>HD-Zip transcription factors</td>
<td>Lateral root development and leaf polarity</td>
</tr>
<tr>
<td>miR171g/b/a</td>
<td>Scarecrow-like transcription factors</td>
<td>Floral development</td>
</tr>
<tr>
<td>miR390</td>
<td>Leucine-rich repeat receptor-like protein kinases</td>
<td>Signal transduction; stimulus response</td>
</tr>
<tr>
<td>miR396d</td>
<td>Growth regulating factor transcription factors, rhodanese-like proteins, kinesin-like protein B</td>
<td>Flower and leaf development</td>
</tr>
<tr>
<td>miR156l/k/a</td>
<td>Squamosa-promoter-binding protein transcription factors</td>
<td>Plant phase transition; shoot development</td>
</tr>
<tr>
<td>miR444b.1</td>
<td>MADS-box transcription factors</td>
<td>Unknown</td>
</tr>
<tr>
<td>miR1432</td>
<td>EF-hand proteins</td>
<td>Signal transduction</td>
</tr>
</tbody>
</table>
occurring in the miR156, miR166, miR169, and miR171 families (Supplementary Table S4 at JXB online). To clarify whether the MREs had a highly specific occurrence in the identified Cd-responsive miRNA families (Table 3), all rice miRNA promoters (Supplementary Fig. S2) were included for MRE distribution frequency calculation (Supplementary Table S4). The results indicated that MREs were present more frequently in Cd-related miRNA families than in other miRNA families (0.58 MREs per each Cd-related miRNA promoter versus 0.16 MREs per each additional miRNA promoter). This difference was significant (t-test, P=0.01793). However, not all of the promoters of the Cd-related miRNA genes had MREs (Table 3). One possibility was that there were some metal-responsive cis-elements, other than the known MREs, which were also responsible for heavy metal signal transduction.

In the present study, several potential cis-acting elements responsive to other environmental signals were also identified in the promoters of the Cd-related miRNA genes. As shown in Supplementary Table S5 at JXB online, stress-responsive cis-elements in the promoters of the six validated miRNAs included: ARE (anaerobic-responsive element); ABRE (ABA-responsive element); GARE (gibberellin-responsive element); ERE (ethylene-responsive element); HSE (heat stress-responsive element); and LTR (low temperature-responsive element). All six validated miRNAs had AREs in their promoters, which responded to hypoxic, low temperature, dehydration stress, and submergence conditions (Dolferus et al., 1994; Zhang et al., 2008). The existence of these putative stress-responsive cis-elements suggested that these miRNAs might respond to a variety of environmental signals, including heavy metal stress.

**Discussion**

Heavy metal pollution is an increasing environmental problem, and Cd, in particular, is extremely toxic to plants. To overcome such toxicity, plants use various strategies to adapt to Cd stress. Numerous studies have investigated the mechanisms of Cd tolerance of plants (e.g. Clemens, 2001; Hall, 2002). Recently, miRNAs emerged as key players in plant responses and adaptation to heavy metal stress (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Lu et al., 2005). Thus, whether miRNAs were involved in Cd tolerance in plants became
one of the essential issues to be addressed. In this study, miRNA microarray assays led to the identification of 19 Cd stress-responsive miRNAs in rice. Interestingly, most of these miRNAs were down-regulated, suggesting that expression of their target genes was turned on in response to Cd stress. In *M. truncatula*, miR166 was reported to be down-regulated by 80 μM Cd, whereas miR171 was up-regulated by 80 μM Cd (Zhou et al., 2008). However, in this study, both miR166 and miR171 in rice were down-regulated in response to Cd treatment. The differential expression pattern of miR171 could be attributed to the different Cd treatment conditions and/or plant species. In addition, miR156, miR171, and miR396 were reported to be down-regulated by 80 μM Cd in *B. napus* (Xie et al., 2007). In *Arabidopsis*, miR168, miR171, and miR396 were all regulated by abiotic stresses such as salinity, drought, and cold (Liu et al., 2008). These results implicated that miR166, miR156, miR171, miR168, and miR396 were most likely to be involved in the plant response to abiotic stresses, including heavy metal stress. However, their exact roles remained to be verified in future experiments, such as overexpression or inhibited expression of these miRNAs.

Microarray data showed that the expression levels of miR168, miR528, and miR162 changed significantly under Cd stress, and their target genes were reported to be involved in miRNA biogenesis. miR528 and miR162 were already known to target the gene encoding the RNA slicer enzyme DCL1, whereas miR168 was reported to target AGO1, a key component of the RISC complex in the miRNA pathway (Vazquez et al., 2004). Thus, the significantly altered expression of these three miRNAs (miR528, miR162, and miR168) suggested that negative feedback regulatory circuits of the miRNA processing/action pathways might be highly active during Cd stress. It has been generally assumed that miRNA expression inversely correlated with target transcript accumulation. However, recent reports have revealed more complex scenarios for miRNA-guided regulation of gene expression in plants, and the target gene can control miRNA accumulation besides being regulated by it (Vaucheret et al., 2006; Sire et al., 2009). The present results are compatible with this model, since a non-perfect complimentary pattern between miR168 and *AGO* in rice roots during Cd treatment was observed (Fig. 3A). The higher level of miR168 did not coincide with the lower accumulation of *AGO* at the 12 h time point. These results suggested the complexity of miRNA/target regulation.

According to the characteristics of the predicted targets and their functions, the Cd-responsive miRNAs had a strong propensity to function in plant development, signal transduction, and abiotic stress responses. A variety of transcription factors were identified as the targets of Cd-related miRNAs. For example, HD-Zip transcription factors targeted by miR166 were involved in shoot meristem initiation, leaf polarity establishment, and lateral root development (Hawker and Bowman, 2004; Prigge et al., 2005). The HD-Zip transcription factors in *Arabidopsis* were also reported to be responsive to water deficiency, osmotic stress, and exogenous ABA treatment (Henriksson et al., 2005). In rice, the HD-Zip family members were induced by various environmental stimuli, including drought and phytohormones (Agalou et al., 2008; Dai et al., 2008). Liu et al. (2009) reported that miR166 in rice was down-regulated by gibberellin (GA), followed by the elevated expression level of HD-Zip. Consistently, in this study, miR166 was down-regulated, whereas the expression of a HD-Zip gene was induced in rice roots exposed to 60 μM Cd for 0–24 h (Fig. 3B). This negatively correlated expression pattern between miR166 and HD-Zip further validated the regulatory role of miR166 in HD-Zip expression. In this experiment it was shown that many targets of Cd-responsive miRNAs encode various components of signal transduction pathways. For example, miR1432 was predicted to target EF-hand family proteins. The EF-hand domain can bind a single Ca^{2+} molecule, which mediates signal transduction pathways in plants (Lu et al., 2008; Sunkar et al., 2008). The prediction of the Ca^{2+} signal transduction component suggested a role for miR1432 in Ca^{2+} signalling and a link between Cd and Ca signalling. In addition, miR390 was predicted to target an LRR-like protein kinase (RLK) (*LOC_Os02g10100*). The RLK gene was previously experimentally verified to be the target of miR390 by 5′ rapid amplification of cDNA ends (RACE; Sunkar et al., 2005). LRR-RLKs contain an extracellular LRR and a Ser/Thr kinase domain, and can activate a complex array of intracellular signalling pathways in response to various plant environmental and developmental signals (Tichtinsky et al., 2003; Osakabe et al., 2005). Plant LRR-RLKs were reported to be involved in multiple processes: ERECTA, HAESA, and CLV1 in development; Xa21 in disease resistance; brassinosteroid-insensitive-1 (BRI1) in steroid hormone signalling; and RPK1 in ABA signal transduction (Friedrichsen et al., 2000; Osakabe et al., 2005). However, LRR-RLK-mediated pathway(s) involved in heavy metal tolerance have not been well understood in plants. In the present study, expression of RLK in rice seedlings exposed to Cd was negatively regulated by miR390 (Fig. 3C). The results confirmed that this kinase gene was targeted by miR390 and indicated that the RLK cascade(s) might function in Cd signalling in rice. Further research on LRR-RLK cascades in rice will promote understanding of the signalling pathways under heavy metal stresses.

**Conclusion**

In the present study, miRNA microarrays were used to profile the expression patterns of annotated miRNAs (miRBase Release 11.0; http://www.mirbase.org/) in rice roots under Cd stress, leading to the identification of 19 Cd-responsive miRNAs. Among them, 18 miRNAs were down-regulated and only miR528 was up-regulated under Cd stress. Target gene prediction and metal stress-responsive cis-element analysis of the Cd-responsive miRNA promoters
provided further evidence for the potential involvement of these miRNAs in response to Cd stress. The data set of the identified Cd-responsive miRNAs is potentially important for additional characterization of the molecular mechanisms underlying Cd tolerance in rice. However, further functional analysis of Cd-responsive miRNAs is required to confirm their function in heavy metal tolerance in plants.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. RT-PCR analysis of the pre-miRNAs.

Table S1. Cis-responsive miRNAs by microarray analysis.

Table S2. The stem–loop RT-PCR primer sequences.

Table S3. Primers of RT-PCR for pre-miRNAs.

Table S4. MREs in the promoters of osa-miRNAs.

Table S5. Analysis of the stress-related cis-elements of the six Cd-responsive miRNAs.

Acknowledgements

This work was supported by Zhejiang Provincial Natural Science Foundation of China [Z3100327], the National Nature Science Foundation of China [31071348], the State Key Laboratory of Rice Biology, China National Rice Research Institute [090102], and the 2009 Zhejiang Science Foundation of China [Z3100327], the National Innovation Program for Graduates. The authors thank Dr Yijun Meng (College of Life Sciences, Zhejiang University) for his efforts in improving the language of this article.

References


